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ONTARIO RESEARCH ENHANCEMENT PROGRAM (O R E P)

Final Report

Development of rapid and quantitative procedures for identification of plant pathogenic *Streptomyces* spp. from soil

I. Detection using PCR primers for *nec1* virulence locus
II. Identification using cellular fatty acid profiles

REPORT No. OREP - 1999/09

LEAD INVESTIGATOR
George Lazarovits

Canada

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November 2001

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FORWARD

The Ontario Research Enhancement Program (OREP) is a $4-million two-year federal research initiative that is administered by the Research Branch of Agriculture and Agri-Food Canada (AAFC), with input from the agriculture and agri-food sector, universities and the province. Research focuses on two areas identified by the sector as:

1. Priorities responding to consumer demand for higher quality products; and
2. Ensuring crop-production management systems are environmentally sustainable.

Biotechnology is one of the areas to be explored. Ontario's agricultural production is based primarily on growing diversified crops using intensive production practices, but long-term viability is linked to the development of sustainable crop production management systems.

There are also opportunities to enhance the economic contribution of Ontario's agriculture and agri-food sector by adding value to the diversified primary commodities produced in the province. The Program is expected to be of particular interest to the corn, soybean, greenhouse, fruit and vegetable sectors and the emphasis will be on projects focussing on food quality improvement and sustainable crop production management such as:

1. The development of value-added animal and crop food products and ingredients that meet domestic and export market demands, with a particular emphasis on quality issues such as processing, packaging, shelf life, new product and ingredient development, high-value alternative uses, probiotics and functional foods;
2. Biotechnologies to reduce environmental pressures related to crop protection practices; and,
3. Integrated crop protection and production practices for both field and greenhouse crops.

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EXECUTIVE SUMMARY

The only procedure available for evaluation of a treatment for reducing the incidence of potato scab, caused by the bacterium *Streptomyces scabies*, is field experimentation. This is both expensive in time and labour, and greatly limits the number of experiments that can be carried out. To accelerate the process for developing a disease control strategy, technology that allows for the determination of populations of pathogenic *S. scabies* from soil was required. This study evaluated if the Polymerase Chain Reaction (PCR) could be used to detect pathogenic *S. scabies* in soil. For this we utilized PCR primers from a region of *S. scabies* DNA (*nec1*) associated with virulence. We also determined if fatty acid (CFA) profiles could be used to differentiate *Streptomyces* species and if so, to create a data base for future identification of these organisms.

The results of this work demonstrated that DNA extraction from soil and *nec1* PCR amplification were very reproducible. PCR was specific for pathogenic *S. scabies* strains 9 out of 10 times, given that the *nec1* region is truly a virulence marker. The intensity of *nec1* PCR amplification from pathogenic *S. scabies*-spiked soil was found to be correlated with the number of bacteria added to the soil. Thus, this test would provide a technique that could be relied upon with confidence for making comparisons of the amount of pathogenic *S. scabies* DNA in soil samples. In addition, DNA was readily extracted from soils of several different soil types and the result for predicting populations found to be equally efficient. To this point, however, we have only tested a limited number of soils and success for detected pathogenic *S. scabies* cannot be predicted for all soils on a universal basis.

A PCR-ELISA method was found to be 2- to 4-times more sensitive for detection of *nec1* PCR product than PCR alone. The procedure is highly promising as a rapid means of quantifying pathogenic *S. scabies* from soil. At this time, however, the variability within replicates in identical experiments with PCR-ELISA was found to be greater than that found using PCR alone. The source of the variation is under investigation.

Use of CFA profile analysis to identify *Streptomyces* spp. is a viable alternative to classical morphological methods. The CFA profiles of *S. scabies* strains were stable and repeatable for the same isolate when established conditions were used. Variation in the CFA profile of the same isolate over time was minimal. However, a more extensive library needs to be established for routine identification of this species of bacteria.

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OBJECTIVES

1) To develop molecular techniques for identification and enumeration of plant pathogenic *Streptomyces scabies* from soil.
2) To determine if cellular fatty acid analysis can be used to differentiate *Streptomyces* species and to create a data base using fatty acid methyl ester information.

PART I

Detection of plant pathogenic *Streptomyces scabies* from soil using PCR primers for *nec1* virulence locus

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INTRODUCTION

Potato scab is caused by several pathogenic *Streptomyces* spp. with *S. scabies* being the most important species (Loria et al. 1997). *S. acidiscabies* is the main causal agent in soils below pH 5 (Loria et al. 1997). Pathogenic *Streptomyces* strains produce phytotoxins named thaxtomins which elicit symptoms of potato scab and are closely associated with virulence (Conn et al. 1998, King et al. 1991, Loria et al. 1995). Depending on the *Streptomyces* strain and the environment, shallow, raised, or deep-pitted lesions develop (Loria et al. 1997). Little is known about the ecology of this pathogen in soil because there is no quick and easy method of detecting it in soil. A semi-selective medium for detecting *Streptomyces* bacteria has been developed (Conn et al. 1998). On this medium, all *S. scabies* strains that were isolated from potato scab lesions produced a melanin pigment and did not sporulate within 2 weeks at 24 °C. These strains also produced thaxtomin indicating that they were pathogenic. However, when bacteria of this phenotype were isolated from soil, only a small percentage produced thaxtomin (Conn et al. 1998). This confirmed previous findings that only a small percentage of *S. scabies* can produce thaxtomin and incite disease (Keinath & Loria 1989). Thus, to determine the proportion of pathogenic to non-

pathogenic phenotypes within a soil population, one would have to examine hundreds of individual colonies for their capacity to produce thaxtomin. This is very time-consuming and not conducive to ecological studies. We therefore examined if the Polymerase Chain Reaction (PCR) could be used to detect pathogenic *S. scabies* in soil. PCR primers developed from a region of *S. scabies* DNA (*nec1*) that is associated with virulence (Bukhalid & Loria 1997) were used. We started by determining the specificity and sensitivity of these PCR primers to detect DNA isolated from thaxtomin-producing (thax$^+$) and non-thaxtomin-producing (thax$^-$) *Streptomyces* strains. The efficiency and reproducibility of several protocols for extraction of DNA out of soil were then determined. The reproducibility and sensitivity of the PCR assay to detect the *nec1* gene was then tested using soil spiked with a pathogenic *S. scabies* strain as well as naturally infested field soils. Several PCR-ELISA protocols for the detection of the *nec1* gene were also examined to determine if sensitivity of *nec1* detection and the efficiency of testing a large number of samples could be improved.

MATERIALS AND METHODS

Streptomyces strains and media

The *Streptomyces* strains used in this study are listed in appendix A and the source of these strains is given in appendix B. Strains were grown on yeast malt extract (YME) medium (Loria & Davis 1988) for spore production and stored as spore suspensions in 15% glycerol at -20 °C. *Streptomyces* strains were grown on Streptomyces selective (STR) medium (Conn et al. 1998) for colony hybridization experiments and for enumeration of *Streptomyces* from soil.

Soils

The soils used in this study were from Alliston, Ontario (site B), London, Ontario (site L), Calgary, Alberta, and Prince Edward Island. The textures of these soils were loamy sand, loam, black loam, and red sand, respectively. Some soil samples (B1A, B1B, B1C, B9A, B9B, B9C) from site B, which had been characterized with respect to the number of pathogenic *S. scabies* per gram of soil, were used in an experiment to determine if PCR could detect different amounts of *S.*

scabies in soil. The number of pathogenic *S. scabies* in these soils had been determined according to Conn et. al. (1998) and were <2000, 2000, 27,000, 42,000, 47,000, and 213,000 colony-forming units (CFU)/g soil for B1B, B1C, B1A, B9B, B9A, and B9C, respectively.

Extraction of DNA from *Streptomyces* bacteria

Streptomyces spores were inoculated into 2 ml YME broth and incubated for 3 d at 28 °C on a shaker at 200 rotations per minute (rpm). One ml of cell suspension was centrifuged for 5 min at 15,000 x g. The cells were washed twice in 1 ml sterile water by repeated centrifugation for 5 min at 15,000 x g, resuspended in 100 µl sterile water, the suspension boiled in a water bath for 10 min, the cell debris pelleted by centrifugation for 1 min at 15,000 x g, and the supernatant used for PCR. A 10 µl aliquot was used for each 25 µl PCR reaction.

Extraction of DNA from soil

Microcon filter method. DNA was extracted from soil according to Porteous et al. (1997) with some modifications. Five hundred mg of air-dried soil were placed into a 2 ml Eppendorf tube containing 925 µl sodium dodecyl sulphate (SDS) lysis buffer (0.25 M NaCl, 0.1 M ethylenediaminetetraacetic acid (EDTA), and 4% SDS) and 75µl guanidine isothiocyanate. The tube was vortexed for 1 min and sonicated for 2 min in an ultrasonic waterbath to disrupt the soil particles and cells within. The sample was then incubated at 68 °C for 1 h to completely lyse the cells and centrifuged at 15,000 x g for 15 min at 4 °C. Six hundred µl of the supernatant was transferred to a new tube and the DNA in the suspension precipitated by addition of 75µl 5 M potassium acetate, 250 µl 40% polyethylene glycol 8000, and 10 µg glycogen for at least 1 h at -20 °C. The suspension was centrifuged at 15,000 x g for 15 min at 4 °C. The pellet was dissolved in 900 µl of 2xCTAB (2% cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, and 0.1 M EDTA) with sonication for 1 min. The sample was then incubated at 68°C for 15 min and 900 µl of chloroform was added to the tube. The tube was gently mixed and centrifuged at 15,000 x g for 10 min at room temperature. The aqueous phase was transferred to a new tube and the DNA precipitated with 1 ml isopropanol for 1 h at -20 °C and pelleted by centrifugation at 15,000 x g for 15 min at 4 °C. The pellet was then dissolved in 450 µl 2.5 M ammonium acetate and 1 ml 95%

ethanol was added to precipitate the DNA at -20 °C overnight. The pellet was vacuum-dried, resuspended in 450 µl 1x tris-acetate/EDTA buffer (TAE; 0.04 M Tris-acetic acid, pH 8.0, 0.001 M EDTA), and transferred to a microcon-30 or microcon-100 tube (Amicon Inc., Beverly, MA 01915, USA). The tube was centrifuged for 24 min, the membrane washed with 450 µl of 1xTAE twice, and with TE (0.01 M Tris-HCl, pH 8.0, 0.001 M EDTA) once to separate DNA from the residual impurities. The sample was finally adjusted to 200 µl with TE and 4 µl sample used as soil DNA extracts for each 25 µl PCR reaction.

Bio 101 kit method. DNA was extracted from soil (50-500 mg) using the Bio 101 FastDNA spin kit as described by Borneman et al. (1996) with modifications. Following disruption of the soil in 1 ml buffer using a FastPrep machine (Bio101, Savant Bio/Can Scientific, Toronto, Ontario), 0.7 ml of supernatant was removed and mixed with 250µl protein precipitation solution (Cat# 6540-403). This mixture was centrifuged at 20,000 x g for 5 min and the supernatant mixed with the binding matrix in a new Eppendorf tube. Tubes were gently inverted for 2 min and allowed to settle for 3 min prior to centrifugation with a Spinfilter for 1 min at 20,000 x g. The pellet was washed twice with 500 µl wash solution (Cat# 6540-405) and centrifuged for 2 min to dry the pellet. To elute the DNA, the Spinfilter was placed into a new catch tube, 100 µl elution solution was then added (Cat# 6540-406), and it was then centrifuged for 1 min at 20,000 x g. The eluted DNA was diluted 10-fold with TE. Two µl of the diluted samples were used for each PCR reaction. Milk quenching was done according to (Volossiouk et al. 1995).

PCR reaction

The primers N_2 (5' GCGCCATATGAGCGCGAACGGAAG-CCCCGGA 3') and N_3 (5' GCGCGAATTCGCAGGTCGTCACGAAGGATCG 3') were made according to Bukhalid & Loria (1997). Primers Y_1/Y_2 were selected from the N_2 and N_3 DNA region. Their sequences were: Y_1 (5'GTGACGCCAAAGACTCAATCAAGTTC3') and Y_2(5'CATATAGCAAC CGACGACACCTGGTC 3'). For each 25 µl PCR reaction, there were 5 µl 5x Gitschier buffer (Kogan et al. 1987), 0.2 µl BSA, 1.15 µl H_2O, 1.25 µl DNTPs mix (25mM ATP:GTP:CTP:TTP, 1:1:1:1), 2 µl DMSO (not used with extracts from *Streptomyces*), 1 µl of primer N_2 (50 pmol/µl),

1 µl of primer N_3 (50 pmol/µl), 0.4 µl of Taq polymerase (2 U Perkin/Elmer), and 2 µl soil DNA extract. The PCR reaction for *Streptomyces* spore extracts was carried out as follows: denaturation at 96 °C for 1 min, annealing at 65 °C for 1 min, and polymerization at 72 °C for 2 min for 30 cycles with a Personal Cycler (Fisher Scientific). For soil DNA extracts, dimethyl sulfoxide (DMSO) was added in the reaction with the following conditions: denaturation at 96 °C for 30 sec, annealing at 65 °C for 30 sec, and polymerization at 72 °C for 2 min for 50 cycles with a GeneAmp 9700 (Perkin Elmer).

Visualization of DNA on agarose gel

Ten µl of PCR product was mixed with 2 µl of 5x loading buffer and loaded onto a 1.2% agarose gel containing ethidium bromide (500 ng/ml). The gel was run at 80 volts for 1.5 h. The electrophoresis gel image was taken under ethidium bromide saturation. The intensity of *nec1* bands was converted into a number by measuring the intensity of the fluorescence of the band at six locations within the band with a cursor using the Quantity One software (BioRad, version 4.11). Background intensity was also measured and subtracted from the readings for the bands. The means of three replicate bands (n = 18) and standard error were calculated and graphed using Sigma Plot (Jandel Scientific).

Competitive PCR

A known sequence (480 base pairs, appendix C) of Vaccinia virus (strain Lister, kindly supplied by Dr. G. McFadden, Robarts Research Institute, University of Western Ontario, London, ON)was amplified with composite primers (5' GCGCCATATGAGCGCGAACGGAAGCCCCGGA CCAATTATGAAACAATAT3' and 5'GCGCGAATTCGCAGGTCGTCACGAAGGATCGTCGCG GGAGAGTCCTTAC 3'). The amplified product was used as an internal standard for competitive PCR.

Colony hybridization

Streptomyces bacteria were grown on STR medium for 2 days at 28 °C, a wetted filter paper over-laid on the agar plates, incubated for 4 more days at the same temperature, the filter papers

lifted, treated, and hybridized with digoxigenin (DIG)-labeled *nec1* probe according to Boehringer-Mannheim protocol (The DIG System User's Guide, pp. 51-60).

Effect of soil texture on DNA extraction from soil

Soils from Ontario (site L), Alberta, and PEI were autoclaved for 1 h, allowed to air-dry in a laminar flow hood, 5 and 50 µl of culture medium containing approximately 20 CFU of *S. scabies* strain SP per µl were added into 500 mg soil giving concentrations of 200 and 2000 CFU/g soil. The mixture was incubated at 4 °C overnight before DNA was extracted (Bio 101 kit) and *nec1* PCR amplified competitively with the internal standard virus DNA. This experiment was carried out twice.

Sensitivity of PCR detection of *nec1* from *S. scabies* spores/mycelium

Genomic DNA of *S. scabies* strain SP was 10-fold serial diluted (from 30 ng to 0.3 ng) and amplified using the PCR protocol described above. This experiment was carried out three times.

Reproducibility and sensitivity of PCR detection of *nec1* from soil

Soil from site B was autoclaved for 1 h and then air-dried in a laminar flow hood. A sample of this autoclaved soil was infested with *S. scabies* strain SP and serial 2-fold dilutions made with non-inoculated sterile soil. 500 mg of the diluted samples were then extracted with the Bio101 kit and extracts PCR amplified. Three replicates of each dilution were made and each replicate was tested in triplicate for PCR product. This experiment was carried out twice.

PCR-ELISA detection of *nec1*

Dig method. PCR-enzyme-linked immunosorbant assay (ELISA) detection was carried out with the Boehringer Mannheim PCR-ELISA kit (Cat# 1636111) according to the protocol supplied (The DIG System User's Guide, pp. 51-60). DNA of *S. scabies* strain SP was used for PCR with DIG-labeled dNTPs. A serial dilution of *nec1* PCR product was then used for ELISA.

Tropix method. PCR-ELISA detection was carried out according to the Tropix method (TROPIX catalog, 1998-1999, p. 26). *Nec1* PCR product was amplified with N_3 and 5' biotin-

labeled N_2 primers from DNA of *S. scabies* strain SP. The product was 2-fold serially diluted with water, 10 µl of each dilution mixed with 20 µl of denaturation buffer for 20 min, 220 µl of fluorescein-labeled probe (Fluorescein GCAAATCCTTC-CGCTGCGTTCGCCA) (7.5 pM in hybridization buffer) added to the tube, and mixed. A 200 µl aliquot of the mixture was transferred to streptavidin-coated microtiter well and hybridized for 3 h at 37 °C. After hybridization, the microtiter wells were washed 5 times with wash buffer, 200 µl antifluorescein antibody conjugated with peroxidase (150 milli units) in conjugate buffer added, and incubated for 30 min at 37 °C. The wells were washed with wash buffer 5 times and 200µl 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate (6)] diammonium in substrate buffer added. Product formation was for 30 min at 37 °C and the colour intensity was determined at 405 nm with an ELISA plate reader (BioRad).

Modified Tropix method. The above procedure was modified as follows: N_2 primer was labeled with fluorescein and the probe was labeled with biotin. At first N_2/N_3 pair were used, and later on Y_1/Y_2 pair (with Y_1 labeled with fluorescein) were used. *Nec1* PCR product from DNA of *S. scabies* strain SP isolated from both spores and site B soil spiked strain SP were tested.

Comparison of sensitivity between PCR-agarose gel and PCR-ELISA methods

Nec1 PCR product amplified with Y_2 and fluorescein labeled Y_1 using DNA of *S. scabies* strain SP was 2-fold serially diluted with water. An aliquot of 10 µl of samples of dilutions was loaded either onto an agarose gel or into streptavidin-coated microtiter wells (modified Tropix method) for electrophoresis or PCR-ELISA, respectively. This experiment was carried out twice.

RESULTS

Specificity of primers for *nec1*

N_2/N_3. Genomic DNA was extracted from two pathogenic *S. scabies* strains (RL 84-01-232 and SP) and a non-pathogenic *Streptomyces* strain M2A-3. The first primer pair used was N_2/N_3 developed by Bukhalid & Loria (1997). This primer pair amplified a single DNA fragment (~700 base pairs, called *nec1* gene) from DNA from the two pathogenic strains but no amplification product was observed from the non-pathogenic strain (data not shown). Universal primers such as BOX and ERIC amplified DNA from all three strains (data not shown).

Y_1/Y_2. Several other primer pairs developed from the same region of DNA as N_2/N_3 were also tested. Only one pair of primers (Y_1/Y_2) could prime the *nec1* amplification with lower annealing temperature (55 °C in comparison to 65 °C for N_2/N_3) without losing the specificity and yield (data not shown). The product of Y_1/Y_2 primers was slightly smaller (~500 base pairs) than N_2/N_3 paired product. All other primers either produced more than one product or produced one product with poor yield (data not shown). Since Y_1/Y_2 gave similar specificity and yield with lower annealing temperature, they were used to replace N_2/N_3 in most of the experiments in this study.

Specificity of *nec1* probe for *nec1*

Colony hybridizations using *nec1* as a probe was tested to determine if this procedure could specifically detect pathogenic *S. scabies*. Pathogenic *S. scabies* strains RL 84-01-232 and SP along with a non-pathogenic *Streptomyces* strain M2A-3 were used. Positive hybridization signals were observed with all three bacteria (Fig. 1). Changing the stringency of the hybridization did not differentiate between the pathogenic and non-pathogenic strains.

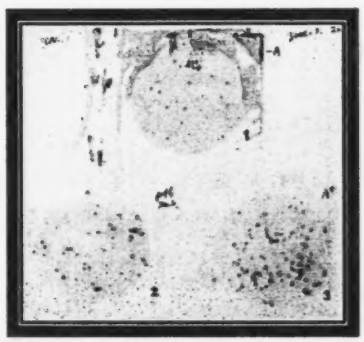

Fig. 1. Specificity of *nec1* probe for *nec1*. Colony hybridizations were carried out with *nec1* probe for non-pathogenic (M2A-3, filter 1) and pathogenic (SP, filter 2; and RL 84-01-232, filter 3) *S. scabies* strains.

Correlation between thaxtomin production and presence of *nec1* in some *Streptomyces* strains

One hundred eighteen *Streptomyces* strains that had been characterized with respect to thaxtomin production (appendix A) were tested for the presence of *nec1* using PCR. Fifty six of these strains produced a PCR product with the *nec1* primers and were thus considered *nec1*$^+$ (Table 1). Of these, 45 were thax$^+$ and 11 were thax$^-$ (Table 1). The pathogenicity of the 11 *nec1*$^+$ thax$^-$ strains has not been determined. Of the 62 isolates that did not react with the *nec1* primers (*nec1*$^-$), only one was thax$^+$ (Table 1). This thax$^+$ *nec1*$^-$ isolate was *S. scabies* strain LR90-3 (appendix A) obtained from the culture collection of C.H. Lawrence and R.R King (appendix B). *Nec1* was not detected by the PCR reaction for strain LR90-3 but it was detected when DNA from strain LR90-3 was hybridized with the *nec1* probe on a Southern blot (data not shown).

Table 1. Correlation between thaxtomin production and presence of *nec1* gene in some *Streptomyces* strains[a].

Thaxtomin biosynthetic ability[b]	PCR detection of *nec1*[c]	
	Nec1$^+$	*Nec1*$^-$
Thax$^+$	45	1
Thax$^-$	11	61

[a] A list of all strains tested and the individual results is given in appendix A.
[b] Thax$^+$ and thax$^-$ refer to strains which did or did not produce thaxtomin on oatmeal medium, respectively.
[c] *Nec1*$^+$ and *nec1*$^-$ refer to strains for which the *nec1* gene could or could not be amplified by PCR, respectively.

Comparison of methods for extraction of DNA from soil

Both the microcon and Bio101 methods produced PCR ready DNA. The yields of PCR product from soil extracts using these methods were similar and typically only one band was obtained. However, sometimes one or two secondary PCR products were visible but these could not be confused with the *nec1* band because the extra bands were smaller in size (data not shown). Modifications, such as including milk to quench potential PCR inhibitors or using microcons with different molecular weight cut-offs, did not improve DNA extraction with regard to PCR yield or the specificity of PCR reaction (data not shown). Extraction of soil quantities between 400 to 500 mg produced the best PCR product yield.

Reproducibility of DNA extraction from soil

Soil from site B was infested with five different amounts of *S. scabies* strain SP spores. Three replicate DNA extractions were then made from each soil to determine how reproducible the DNA extraction procedure was. These soil extracts were then used for PCR. The intensity of the three *nec1* bands for each set of PCR reactions were essentially identical to each other for all sample sets except for the soil with the lowest number of *S. scabies* (Fig. 2). In this set, one band was fainter than the other two (Fig. 2). When the relative intensity of the bands was converted into numbers and the values averaged, small error bars were observed for all sample sets (Fig. 3) demonstrating that DNA extraction from soil was reproducible. There was a linear relationship between band intensity and number of *S. scabies* in soil except for the soils with low numbers of bacteria (Figs. 2, 3).

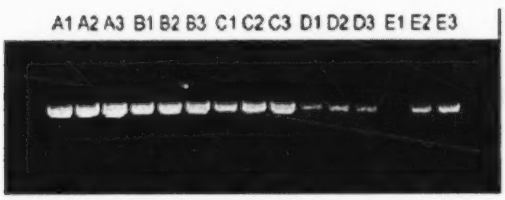

Fig. 2. Reproducibility of DNA extraction from soil. Three replicate DNA extracts were made from site B soil containing (A) 210,000, (B) 42,000, (C) 21,000, (D) 4200, or (E) 2100 *S. scabies* strain SP (CFU/g soil). PCR was then carried out and *nec1* visualized on an agarose gel.

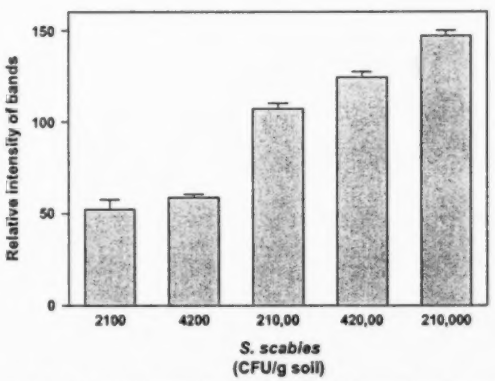

Fig. 3. Relative intensity of *nec1* bands from the agarose gel shown in Figure 2 verses number of *S. scabies* strain SP. Band intensity was determined using a computer program. Means ± SE, n = 18.

Reproducibility of the PCR reaction

Soil from site B was infested with five different amounts of *S. scabies* strain SP spores and then DNA was extracted from each soil sample. Three replicate PCR reactions were then conducted with each soil extract to determine how reproducible the PCR reaction was. The intensity of the three *nec1* bands for each set of PCR reactions were essentially identical to each other (Fig. 4). When the relative intensity of the bands was converted into numbers and the values averaged, small error bars were observed for all sample sets (Fig. 5) demonstrating that the PCR reaction was reproducible. There was a linear relationship between band intensity and number of *S. scabies* in soil except for the soils with low numbers of bacteria (Figs. 4, 5).

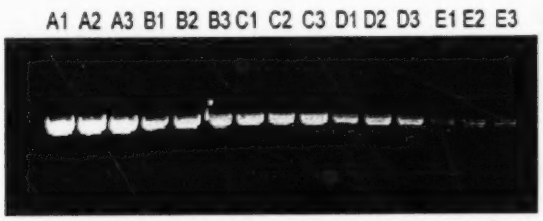

Fig. 4. Reproducibility of the PCR reaction. Three replicate PCR reactions were made using extracts from site B soil containing (A) 210,000, (B) 42,000, (C) 21,000, (D) 4200, or (E) 2100 *S. scabies* strain SP (CFU/g soil). *Nec1* was then visualized on an agarose gel.

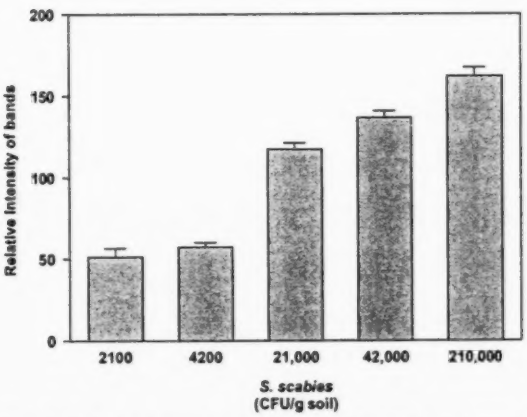

Fig. 5. Relative intensity of *nec1* bands from the agarose gel shown in Figure 4 verses number of *S. scabies* strain SP. Band intensity was determined using a computer program. Means ± SE, n = 18.

Sensitivity of PCR detection of *nec1*

DNA extracted from *S. scabies* spores. Genomic DNA was isolated from *S. scabies* strain SP and a serial dilution made to determine how little *nec1* could be detected by PCR. A *nec1* band was observed in agarose gels from PCR reactions in which as little as 3 pg of template DNA was used (Fig. 6). The relative intensity and size of the bands was directly related to the amount of DNA present in the starting solution (Fig. 6).

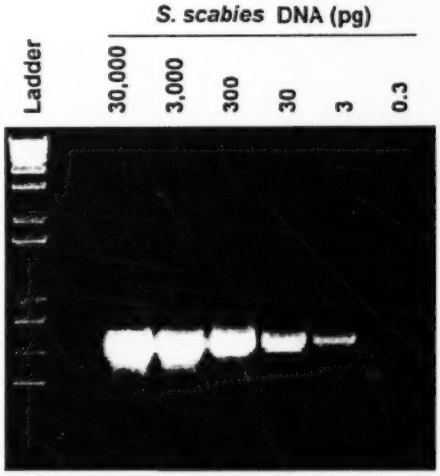

Fig. 6. Sensitivity of PCR detection of *nec1*. Various amounts of genomic DNA from *S. scabies* strain SP was used for the PCR reaction and *nec1* bands visualized on an agarose gel.

DNA extracted from soil. Soil from site B was autoclaved to destroy any natural *S. scabies* population and then infested with *S. scabies* strain SP. The infested soil was diluted with autoclaved soil and soil extracts used for PCR to determine how little *S. scabies* bacteria could be detected. As little as 1600 CFU per gram soil could be detected (Fig. 7). The autoclaved soil was used as a control and failed to amplify *nec1* (Fig. 7). When infested soil was again autoclaved, there was no amplification of *nec1* (data not shown). The PCR reaction produced a gradient of band densities that correlated to the number of *S. scabies* in the soil (Fig. 7).

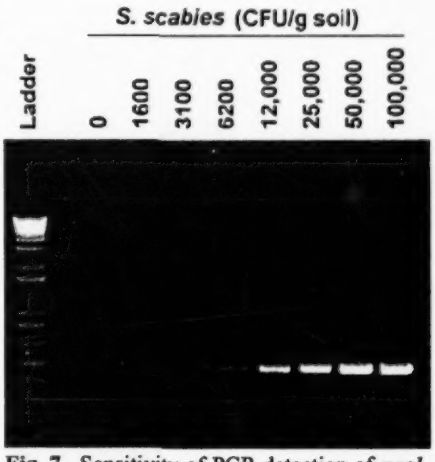

Fig. 7. Sensitivity of PCR detection of *nec1*. DNA from *S. scabies* strain SP infested soils was used for the PCR reaction and *nec1* bands visualized on an agarose gel.

Use of competitive PCR for quantifying *nec1*

Competitive PCR was tested to determine if this could be used as a quantitative tool for *nec1*. This procedure involved mixing increasing amounts of Vaccinia virus DNA with a fixed amount of DNA from *S. scabies* strain SP, carrying out PCR, and visualization of PCR products on an agarose gel. The intensities of the *nec1* and virus bands were most similar in lanes C and D indicating that the amount of *nec1* DNA was between 10^{-6} and 10^{-7} ng (Fig. 8).

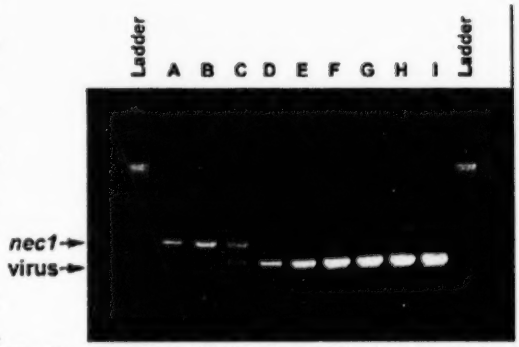

Fig. 8. Competitive PCR using *nec1* primers and virus DNA template. Approximately 50 ng of *S. scabies* strain SP DNA plus 0, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-3}, 10^{-2}, and 10^{-1} ng of virus template DNA (lanes A-I, respectively) were mixed, PCR amplified, and visualized on an agarose gel.

Effect of soil texture on DNA extraction from soil

DNA extraction was initially done with soil from site B which has a loamy sand texture. To determine if extraction efficiency was different for different soil types, several other soils were also tested. Soils from Ontario (site L), Alberta, and PEI, were infested with *S. scabies* strain SP, soil extracts used for PCR, and *nec1* bands visualized on an agarose gel. Virus DNA was used as an internal standard. Similar *nec1* band intensities were detected for all three soil types (Fig. 9). *Nec1* was barely detected for the soils with the lower amounts of bacteria (Fig. 9). Presence of the virus band in all samples indicated that there was no problem with the PCR reaction.

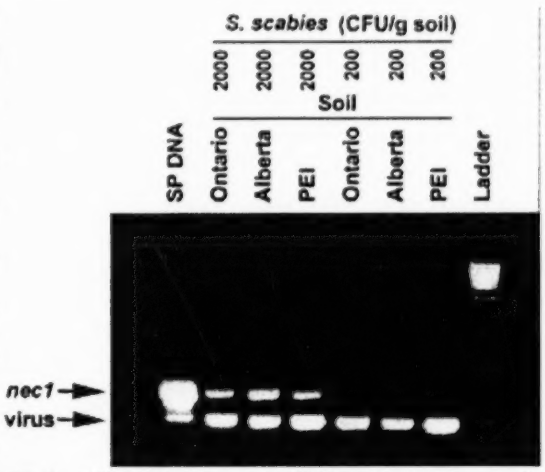

Fig. 9. Effect of soil type on DNA extraction from soil. Soils from Ontario (site L), Alberta, and PEI were infested with *S. scabies* strain SP at 200 and 2000 CFU/g soil. DNA was then extracted, PCR amplified, and PCR products visualized on an agarose gel. An internal standard virus DNA (10^{-6} ng) was included. DNA from *S. scabies* strain SP was included as a standard.

PCR detection of *nec1* from field soil

Soil samples from site B, which had been characterized with respect to the number of thax$^+$ *S. scabies* per gram soil, were used for the PCR reaction with *nec1* primers. No *nec1* band was seen for soil B1B, a moderate strength band for soil B1C, and bright bands for the other soils (Fig. 10). This corresponded with *S. scabies* numbers of <2000, 2000, and 27,000-213,000 CFU per gram soil, respectively. The *nec1* band for soil B9C was similar to that of soils B1A, B9A, and B9C (Fig. 10) even though it had more *S. scabies* present. This is because above a certain point, bands become saturated and differences do not show up. In the PCR procedure used for Figure 10, a 10-fold dilution of the soil extracts was used for PCR. When a serial dilution of 2-fold was used for soil B1B, a faint band showed up (data not shown). A *nec1* band could be seen for soil B9C even with a 200-fold dilution (data not shown).

To demonstrate that lack of a *nec1* band for soil B1B (Fig. 10) was not due to a problem with the PCR reaction, a competitive PCR reaction with virus DNA was carried out (Fig. 11). Using the virus DNA as an internal standard resulted in virus bands of similar intensity for all soil samples while *nec1* bands were of various intensities (Fig. 11). This showed that soil B1B did indeed have little or no

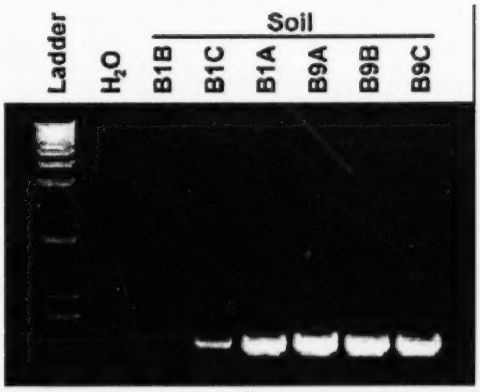

Fig. 10. Detection of *nec1* from naturally infected field soils. DNA from site B soil samples (B1B, B1C, B1A, B9A, B9B, and B9C) for which *S. scabies* numbers had been determined (<2000, 2000, 27,000, 47,000, 42,000, and 213,000 CFU/g soil, respectively) was used in the PCR reaction and *nec1* bands visualized on an agarose gel.

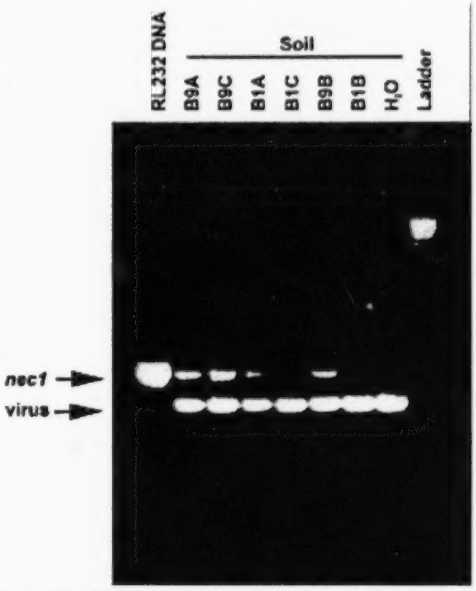

Fig. 11. Verification of PCR detection of *nec1* from naturally infected field soils (same soils as shown in Figure 10) using competitive PCR. Equal amounts of virus DNA (10^{-6} ng) was added to each soil extract before PCR. DNA from *S. scabies* strain RL 84-01-232 was included as a standard.

pathogenic *S. scabies* in it and lack of a band was not because of a problem with the PCR procedure.

Comparison of PCR-agarose gel and PCR-ELISA methods for detection of *nec1*

Several PCR-ELISA protocols were compared to the PCR-agarose gel method to determine if sensitivity of *nec1* detection and the efficiency of testing a large number of samples could be improved. PCR-ELISA was more sensitive for detection of *nec1* than the PCR-agarose gel method. Using the PCR- agarose gel method, about 120 ng *nec1* DNA could be detected (Fig. 12) while PCR-ELISA detected 31 ng DNA (Fig. 13). Of the PCR-ELISA methods examined, the DIG method was the most sensitive (data not shown). However, while PCR-ELISA is more sensitive than the PCR-agarose gel method, it does not give as reproducible results yet (data not shown). All three ELISA methods are faster than the PCR-agarose gel method for testing a large number of samples but are also more expensive.

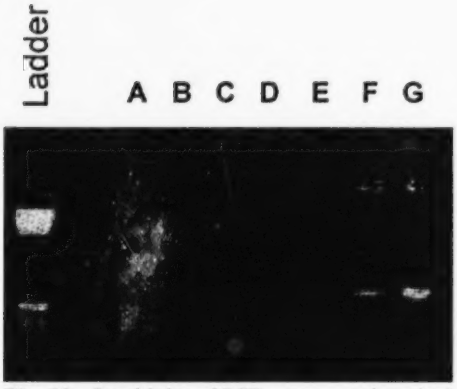

Fig. 12. Sensitivity of PCR-agarose gel method for detection of *nec1*. Approximately 8 (A), 16 (B), 31 (C), 62 (D), 120 (E), 250 (F), and 500 (G) ng of PCR product (*nec1*) were visualized on an agarose gel.

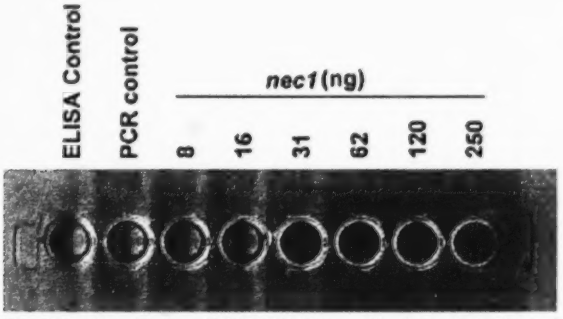

Fig. 13. Sensitivity of PCR-ELISA method for detection of *nec1*. Various amounts of PCR product (*nec1*) were used for ELISA (modified Tropix method).

DISCUSSION

Currently, the efficacy of a treatment such as an organic soil amendment for reducing potato scab is achieved by applying the treatment, planting potatoes, and waiting to examine tubers for scab. This is not only expensive in time and labour, but also greatly limits the number of experiments that can be carried out. To accelerate the process, we needed to be able to determine the population of pathogenic *S. scabies* in soil. However, there was no easy and quick method available to do this. Thus, this study was undertaken to determine if the Polymerase Chain Reaction (PCR) could be used to detect pathogenic *S. scabies* in soil. PCR primers developed from a region of *S. scabies* DNA (*nec1*) that is associated with virulence (Bukhalid & Loria 1997) were used.

Tests on the specificity of the primers to detect pathogenic *S. scabies* showed that of 118 *Streptomyces* strains tested, only one pathogenic strain (LR90-3) was not detected by the primers. This isolate was previously found to lack the primer sequences used for PCR (Loria et al., unpublished data). This strain (LR90-3), however, did hybridize with the *nec1* probe in Southern blot analysis demonstrating that most of the *nec1* gene was present. The fact that only one thax$^+$ strain out of 46 was not detected by PCR is encouraging. This demonstrates that few pathogenic strains would be missed by this assay.

Eleven strains (9%) were found to be *nec1*$^+$ and thax$^-$. The pathogenicity of these strains is not known. However, since these strains have the *nec1* gene, they could be considered to be potentially pathogenic strains. More recent results carried out by J. Kers in our laboratory indicate that quite varied populations in some specific soils may have the *nec1* gene, but not be able to produce thaxtomin. This result does not necessarily negate the use of this assay, however, since this assay did predict pathogenicity at least 90% of the time.

The reproducibility of DNA extraction from soil and of the PCR reaction were found to be very reproducible. Thus, this test would provide a technique that could be relied upon with confidence for making comparisons of the amount of pathogenic *S. scabies* DNA in soil samples. In addition, DNA was readily extracted from soils of several different soil types and the result for predicting populations found to be equally efficient. To this point, however, we have only tested

a limited number of soils and success for detected pathogenic *S. scabies* cannot be predicted for all soils on a universal basis.

As little as 3 pg of DNA from *S. scabies* could be detected. This amount of *nec1* DNA could also be quantified by using competitive PCR methods. The DNA from about 2000 CFU *S. scabies* per gram soil was the limit for accurate and reproducible detection. This may be improved upon by extracting more that 500 mg of soil. We did not see any improvements in detection by using larger volumes of soil extract for the PCR reaction. As the number of pathogenic *S. scabies* increased, we found increases in band intensity of the PCR reaction. Obvious intensity was not apparent above 50,000-100000 CFU. In competitive PCR reactions the competitive probe reduced the detection of low populations of pathogenic *S. scabies,* but did not impede the higher levels. The presence of the competitive reaction product, however, was an excellent positive indicator that the PCR reaction was successfully achieved.

Several PCR-ELISA methods were also examined to determine if they were more efficient and sensitive than the PCR-agarose gel method. They were found to be 2- to 4-times more sensitive for detection of *nec1* PCR product. The DIG PCR-ELISA method was the most sensitive. The procedure is highly promising as a rapid means of quantifying pathogenic *S. scabies* from soil. At this time, however, the variability within replicates and among replicated experiments with PCR-ELISA was found to be greater than that found using PCR alone. The source of the variation is under investigation.

PUBLICATIONS ARISING FROM THIS WORK

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PART II
Identification of plant pathogenic *Streptomyces* spp. using cellular fatty acid profiles

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INTRODUCTION

The members of the genus *Streptomyces* (Family *Actinomycetes*) are ubiquitous, Gram-positive soil bacteria. They develop a true, branched filamentous aerial mycelium and reproduce by spores (conidia) formed in chains at the tips of the hyphae, similar to what is found in the higher fungi. Together with *Nocardia* spp. and *Micromonospora* spp., they are responsible for the musty odour of soil. Many are economically important as plant pathogens causing potato scab, and a root disease in sweet-potato. Not all *Streptomyces* organisms isolated from the soil can cause the common potato scab disease. Those that do are usually grouped collectively under *Streptomyces scabies*. Others produce important antibiotics, e.g., streptomycin by *S. griseus*, chlortetracycline/aureomycin by *S. aureofaciens*, oxytetracycline by *S. rimosus*, chloramphenicol by *S. venezuelae*, neomycin by *S. fradiae*, erythromycin by *S. erythraeus*, and viomycin by *S. puniceus*, *S. floridae*, and *S. vinaneus*. Still others are saprophytic soil bacteria.

Plant pathogenic *S. scabies* have a similar morphological appearance and growth characteristics as non-pathogen species and are difficult to differentiate. Differential methods based on growth and morphological characteristics require about a month to complete. Thus, there is a need to develop more rapid methods to differentiate pathogenic from non-pathogenic strains. The primary objective of this study was to assess the use of cellular fatty acid (CFA) analysis by gas chromatography (GC) to accomplish this task. A second objective was to apply this technique to create a comprehensive CFA profile library for identification of *Streptomyces* spp. Isolates for this study were from the cultural collections of Cornell University and Southern Crop Protection and Food Research Centre (SCPFRC), Agriculture & Agri-Food Canada (AAFC).

The conventional approach in bacterial identification, which is based on cell and colony morphology, cultural characteristics, biochemical and physiological tests, and susceptibility to antibiotics is generally labourious, time-consuming and requires significant training and experience to perform well (Lindholm et al. 1997). Molecular methods are more specific, but also require considerable technical expertise, experience, and time to perform consistently. Over the past two decades, a number of automated systems for identification of microorganisms were introduced, including CFA analysis by GC (Bowers et al. 1996, Ndowora et al. 1996, Schraft et al. 1996, Stager & James 1992, Steele et al 1998, Stoakes et al. 1994, Sasser 1990, Welch 1991). Improvements in GC techniques over this period, together with advances in GC instrumentation and computerization, have led to semi-automated and standardised analytical methods, which simplified the procedure (Brondz & Olsen 1991, Moss 1990). A semi-automated gas chromatographic system for analysis of fatty acids is commercially available from MIDI Inc., DE, U.S.A., making CFA analysis-based microbial identification a practical reality for routine diagnostic and research laboratories. Compared to conventional biochemical and molecular methods, the relative speed and simplicity, low costs in time and materials combine to make this the tool of choice for many laboratories.

MATERIALS AND METHODS

Culture method

Initially, forty-nine, and later increased to a total of 52, *Streptomyces* isolates were received from SCPFRC for CFA profile analysis and classification (Appendix D). These had been grown on potato dextrose agar (PDA) to the sporulating stage. In preparation for CFA analysis by the MIDI-MIS, the isolates were passed twice in 20 ml of sterilized trypticase soy broth (TSB) from BBL®, (concentration: 30 g per litre), in 125 ml Erlenmeyer flasks into which 5 g of 3 mm diameter glass beads had been added. For the first passage cultures, heavy inocula of spores were scraped from PDA and inoculated into the Erlenmeyer flasks. The cultures were incubated in a Labline™ shaker-incubator, Model 4628CC, maintained at 28±1 °C, and shaken at 150 rpm. The cells were allowed to grow to mid-log/beginning of the stationary phase. This usually took 3 to 4 days. For the second passage cultures, 1 ml of the cells from each flask was transferred to fresh TSB and

grown under the same conditions. After 72±1 hours, when the cells were in mid-log phase, the flasks were removed from the shaker-incubator and immediately placed in ice-cold water to slow or stop further growth. They were kept at low temperature until harvested by high-speed centrifugation. Some isolates were observed to grow faster than others and hence arrived at the mid-log phase earlier. But in the interest of standardizing the harvest time, all isolates were harvested after 72±1 hours. Some slow-growing isolates were harvested after four days.

Harvesting and CFA extraction

Glass pasteur pipettes were used to transfer 10 to 15 ml of the bacterial cultures to 30 ml Corex® glass, or Beckman polycarbonate centrifuge tubes. The tubes were balanced in pairs by weight, and pelleted by centrifugation for 10 minutes at 4 °C, at 18,000 x g (12,000 rpm), in a Beckman J2-M1 refrigerated, high-speed floor centrifuge and JA-20 rotor. The supernatant was carefully decanted from each tube. Then, 200±5 mg (wet weight) of bacterial cells were transferred to screw-capped, 13 x 100 mm culture tubes using disposable, sterile inoculation loops. After harvesting, the culture tubes containing the cells were again kept in ice-cold water. If CFA extraction and analysis was not performed within an hour of harvest, the cells were stored in a -86 °C ultra-freezer for up to a maximum of two weeks. Sample preparation for CFA analysis followed the four simple steps described in the MIDI-MIS Operating Manual: 1) CFAs were released from the bacterial cells by saponification with sodium hydroxide-methyl alcohol, 2) methylation to fatty acid methyl esters (FAME) with hydrochloric acid-alcohol, 3) extraction by hexane-MTBE solvent, and 4) the extracts were washed with sodium hydroxide to remove any remaining free fatty acid and transferred to chromatography sample vials.

GC analysis of FAME

CFA profiles analysis was done using the MIDI-MIS running Sherlock® integrated chromatography software (MIDI Inc., Newark, DE, U.S.A.). This system consists of a Hewlett-Packard Series II 5890 Gas Chromatograph (GC) equipped with a 25 m x 0.2 mm phenyl methyl silicone fused silica capillary column, Flame Ionization Detector (FID), 7673A Automatic Sampler, and H-P 3365 ChemStation® Software which provided automated sample handling, controlled GC

parameters, data collection and analysis. The FAME extract vials were loaded in the Automatic Sampler of the H-P gas chromatograph, the isolates entered in the Sample Table by their laboratory identification numbers and analysis started. The results were analysed utilizing Sherlock software through indexing of samples analysed, cluster analysis, pattern recognition and database searches, and in most cases an identification of the organism was made automatically. The 2-D plot and the dendrogram features of the MIDI-Sherlock system were used to determine how closely related or similar the isolates were.

RESULTS AND DISCUSSION

Optimization of culture conditions

The first task of this study was to establish the optimal growth and cultural conditions for the organisms, and an efficient method for harvesting them. While the method described in the MIDI Operating Manual was followed initially, some changes were made. First, the TSB cultures were shaken at 150 rpm instead of the 125 rpm recommended by MIDI, or 225 rpm by Bowers et al. (1996). Growth periods of 3 to 4 days were adopted for the first passage cultures, followed by 72±1 hours for the second passage cultures. Harvest wet weights were increased from the 100 mg recommended, to 150 to 200 mg. The higher figure was ultimately adopted for the course of this work. Lastly, high-speed centrifugation was used for harvesting the cells from TSB cultures instead of vacuum assisted filtration using 45 µm pore-sized membrane filters suggested by MIDI.

In the first phase of this study, all isolates were cultured and analysed as described, and identifications given by MIDI-MIS were recorded. For the majority of isolates tested, the identifications had low Similarity Index (SI) values. The SI is a numerical value expressing how closely the CFA composition of an unknown sample compares with that of the MIDI-MIS database match. Following the MIS convention, SI values of 0.6 to 1.0 indicate an excellent match, 1.0 being the highest; 0.3 to 0.5 is considered very likely to be correct; <0.3 may indicate an organism not in the database but related to the match given. Based on these criteria, more than 70% of the identifications had SI values that were <0.5. This suggested the present MIDI Actin-1 database (Appendix E) for *Actinomycetes*, which contained 24 species, is too limited for identification of this genus. In comparison, the National Center for Biotechnology Information

(National Library of Medicine/National Inst. of Health) in the U.S. listed more than 500 (including 522 named, and 21 unclassified species) *Streptomyces* strains. Bergey's Manual of Microbiology has 150 *Streptomyces* spp. listed. Secondly, part of this discrepancy may also be attributable to the altered growth and environmental parameters, as bacterial CFA composition varies with culture medium, incubation temperature, and physiological age of the cell population. Several investigators had shown that under standardized growth conditions, the same bacterial strain yielded profiles that are highly reproducible and repeatable (Eerola & Lehtonen 1988). In this study, the cultural conditions and medium used were stringently controlled, leaving the differences in the physiological age of the cells as the only factor where there was a potential for variation. This result, in fact, emphasized the need for a more comprehensive CFA profiles-based *Streptomyces* library under the newly established standardized cultural conditions.

Repeatability study

This was done to determine the intrinsic (natural) variability of CFA profiles within the same organism and to check the precision and repeatability of the cultural/harvest/GC-analysis protocol. To do this, five isolates were randomly selected for testing. For 1st passage culture, three colonies from each petri plate culture were inoculated into TSB flasks. For 2nd passage, each was inoculated into two TSB flasks, giving a total of six replicate cultures from each isolate. Three repeat runs were made and dendrogram analysis performed to determine same-isolate variation as well as variation over a period of time. In addition, each isolate profile was also compared with previous results from preceeding analytical runs, effectively extending the comparisons over the duration of this study.

The results of same-batch CFA profiling are shown as dendrograms in Appendix G (a), (b), and (c), for isolates R98-02, R98-09, and R98-12, respectively. Euclidean distances (E-d; Appendix F) were 4.24, 3.91 and 5.31 respectively. In each case the E-d was <6, the cut-off point for sub-species. This result confirmed the method can be used for identifying *Streptomyces* spp.

Appendix H (a), (b), and (c) shows dendrograms of the same three isolates, R98-02, R98-09, and R98-12, and E-d variation over time (i.e. over the duration of the study). E-d values were 8.46,

4.74, and 8.73, respectively, which were slightly above the cut-off (6 E-d) for sub-species, but well within that for species level (10 E-d).

Differentiation of pathogenic and non-pathogenic strains of *Streptomyces*

Besides using the MIS to identify the *Streptomyces* strains, the system was also evaluated for its ability to separate pathogenic and non-pathogenic strains among the samples. The dendrogram (Appendix F) function of MIS software was used for this purpose. Results showed that there was no distinct segregation of these two groups by their CFA profiles, and that while some known pathogens were clustered together, others were distributed at Euclidean distances (Appendix F) that suggested the isolates were not related. In addition, examination of individual fatty acids present in isolates did not provide a conclusive segregation of pathogenic from non-pathogenic strains. However, it was found that CFA profiles of the same isolate cultured and analysed repeatedly over the period of this study, i.e. more than one year, showed very good repeatability and consistency. Dendrograms showed these repeatedly tested organisms grouped at Euclidean distances of <10, which indicated these were the same organism.

ACKNOWLEDGEMENTS

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CONCLUSIONS

1) PCR primers for *nec1* were specific to pathogenic *S. scabies* 9 out of 10 times.
2) PCR provides reproducible results.
3) Extraction of DNA from soil is reproducible.
4) As little as 3 pg of pathogenic *S. scabies* DNA can be detected by PCR.
5) As little as 2000 CFU per g soil of pathogenic *S. scabies* can be detected by PCR.
6) Competitive PCR can be used to quantify *nec1* DNA in soil.
7) Soil type does not effect PCR detection of *nec1*.
8) The intensity of *nec1* PCR amplification from pathogenic *S. scabies*-spiked soil correlates with the number of bacteria added to the soil.
9) The intensity of *nec1* PCR amplification from field soil samples correlates with the number of pathogenic *S. scabies* in the soil.
10) The PCR-ELISA method is more sensitive than the PCR-agarose gel method for detection of *nec1*, but further refinements are required to make it as reproducible.
11) Use of CFA profile analysis to identify *Streptomyces* spp. is a viable alternative to classical morphological methods.
12) The CFA profiles were stable and repeatable for the same isolate when established conditions were used.
13) Variation in the CFA profile of the same isolate over time was minimal.
14) The commercial database from MIDI Inc. was inadequate for routine identification of *Streptomyces* due mainly to the limited number of 24 entries.
15) A more extensive library needs to be established for this species of bacteria.

FUTURE DIRECTIONS

The results from this study indicate that PCR is a simple, inexpensive, and useful tool for determining the presence of virulent pathogens in soil. This is the first study that has attempted to quantify genes for virulence in an ecosystem. The study showed that the PCR reaction can be utilized to determine if populations of pathogenic *S. scabies* have been altered by a specific treatment. We believe that PCR can also be used for predicting disease potential in a specific soil. Considerable more research needs to be carried out, however, to validate the accuracy of this technique for such purposes. Numerous growers and researchers have sent us soil to determine disease potential for next years crop. We do not know if our predictions were found to be correct. Considerable interest has been expressed by several commercial laboratories for exploiting this technique for agricultural use. We have been approached by A&L Laboratories for transfer of this technology for use to diagnose potato soils for potential disease.

This study emphasized the need to establish a more extensive library of cellular fatty acid profiles for *Streptomyces* bacteria. The culture collections of AAFC (London, Ontario, Canada) and Cornell University contain more than six hundred isolates that have already been typed either pathogenic or non-pathogenic by traditional methods. Access to these isolates will be invaluable in expanding the *Streptomyces* library for use in routine identification of this species.

GLOSSARY OF TERMS

deoxyribonucleic acid	DNA
polymerase chain reaction	PCR
thaxtomin-producing	$thax^+$
non-thaxtomin-producing	$thax^-$
enzyme-linked immunosorbant assay	ELISA
yeast malt extract	YME
Streptomyces	STR
colony-forming units	CFU
rotations per minute	rpm
sodium dodecyl sulphate	SDS
ethylenediaminetetraacetic acid	EDTA
cetyltrimethylammonium bromide	CTAB
tris-acetate/EDTA electrophoresis buffer	TAE
dimethyl sulfoxide	DMSO
digoxigenin	DIG
cellular fatty acid	CFA
gas chromatography	GC
potato dextrose agar	PDA
trypticase soy broth	TSB
fatty acid methyl esters	FAME
flame ionization detector	FID

APPENDIX A

***Streptomyces* strains* used in part I of this study.**
Correlation between thaxtomin production and presence of *nec1*.

Strain	Thaxtomin production	Presence of *nec1* gene	Strain	Thaxtomin production	Presence of *nec1* gene
SP	+	+	B-B57	+	+
DP	+	+	B-B58	+	+
PonP	+	+	B-B83	+	+
LPit-2	+	+	B-Sh-1	+	+
LM5MT-2	+	+	B-Sh-3	+	+
RL 84-01-232	+	+	B-Sh-9	+	+
RL 84-34	+	+	KV-D-4	+	+
RL 84-01-174	+	+	KV-D-6	+	+
RL 84-01-110	+	+	KV-Sh-3	+	+
94-001	+	+	M-2C-3	+	+
94-009	+	+	M-7A-16	+	+
94-020	+	+	M-7A-18	+	+
94-025	+	+	M-7A-18'	+	+
94-037	+	+	M-D-2	+	+
94-057	+	+	M-Sh-3	+	+
94-067	+	+	M-Sh-10	+	+
94-070	+	+	P-D-5	+	+
95-115	+	+	P-D-6	+	+
95-122	+	+	P-D-8	+	+
95-154	+	+	P-Sh-7	+	+
95-159	+	+	P-Sh-9	+	+
B-9C-5-15	+	+	S-Sh-B32	+	+
B-B16	+	+			
RL 83-01-03	-	+	M-2D-3	-	+
B-B37	-	+	M-7A-8	-	+
B-B51	-	+	M-7A-10	-	+
B-B54	-	+	M-7	-	+
M-2A-4	-	+	B-8-21	-	+
M-2B-2	-	+			
LR90-3	+	-			

*Source of *Streptomyces* strains is given in appendix B.

continued

***Streptomyces* strains* used in part I of this study continued.**
Correlation between thaxtomin production and presence of *nec1*.

Strain	Thaxtomin production	Presence of *nec1* gene	Strain	Thaxtomin production	Presence of *nec1* gene
Fi II	-	-	M-2A-6	-	-
B-1A-3	-	-	M-2B-1	-	-
B-1A-5	-	-	M-2B-4	-	-
B-1B-4	-	-	M-2C-4	-	-
B-2-1	-	-	M-2D-1	-	-
B-2-4	-	-	M-2D-4	-	-
B-3-2	-	-	M-2D-5	-	-
B-6-2	-	-	M-3A-2	-	-
B-8-6	-	-	M-3A-7	-	-
B-8-7	-	-	M-3B-3	-	-
B-8-8	-	-	M-4A-4	-	-
B-8-12	-	-	M-4A-5	-	-
B-8-13	-	-	M-5C-1	-	-
B-8-14	-	-	M-7A-1	-	-
B-8-17	-	-	M-7A-2	-	-
B-8-19	-	-	M-7A-3	-	-
B-8-20	-	-	M-7A-5	-	-
B-8-23	-	-	M-7A-11	-	-
B-B14	-	-	M-7A-21	-	-
B-B56	-	-	M-7A-22	-	-
B-B82	-	-	M-7A-23	-	-
Co-4Sh-2	-	-	M-7A-26	-	-
Co-4Sh-5	-	-	M-7A-27	-	-
94-008	-	-	M-7A-30	-	-
M-1A-3	-	-	M-7A-31	-	-
M-1B-1	-	-	M-7A-34	-	-
M-2A-1	-	-	M-7A-40	-	-
M-2A-3	-	-	M-9B-4-1	-	-
M-2A-4	-	-	M-12	-	-
M-2A-5	-	-	PR-3-3'	-	-
88CCGR	-	-			

*Source of *Streptomyces* strains is given in appendix B.

APPENDIX B

Source of *Streptomyces* strains used in this study.

Strain	Source
S. scabies	
SP	ON, Canada[b]
DP	MI, USA[c]
Fi II	MI, USA[c]
PonP	MN, USA[d]
Roy	MN, USA[d]
LPit-2	NS, Canada[e]
LR90-3	ON, Canada[e]
LM5MT-2	NB, Canada[e]
RL 84-01-232	USA[f]
RL 84-34	USA[f]
RL 83-01-03	USA[f]
S. acidiscabies	
RL 84-01-174	USA[f]
RL 84-01-110	USA[f]
S. scabies phenotype[a]	
all others	ON, Canada[g]

[a] These strains had the same colony phenotype as *S. scabies* when grown on STR medium so many are likely *S. scabies* but species was not confirmed.
[b] Obtained from the culture collection of G. Lazarovits. *S. scabies* strain SP is pathogenic on potato and produces thaxtomin (Conn et al. 1998).
[c] Obtained from the culture collection of F. Spooner (Michigan State University).
[d] Obtained from the culture collection of N. Anderson (University of Minnesota) via F. Spooner.
[e] Obtained from the culture collection of C.H. Lawrence and R.R. King (AAFC, Fredericton, NB, Canada).
[f] Obtained from the culture collection of R. Loria.
[g] Obtained from the culture collection of G. Lazarovits. Strains were isolated from tuber lesions and soils collected from commercial potato fields in Ontario, Canada.

APPENDIX C

Partial sequence of vaccinia virus (strain Lister) used for internal standard of competitive PCR. Underlined sequences are the primer portions used to amplify this internal standard.

gcaattatgaaacaatatatcgtcctggcatgcatgtgcctggcggcagctgctatgcctgccagtcttcagcaatcatcctcatcctcctcct

cgtgtacggaagaagaaaacaaacatcatatgggaatcgatgttattatcaaagtcacaaagcaagaccaaacaccgaccaatgataagat

ttgccaatccgtaacggaaattacagagtccgagtcagatccagatcccgaggtggaatcagaagatgattccacatcagtcgaggatgta

gatcctcctaccacttattactccatcatcggtggaggtctgagaatgaactttggattcaccaaatgtcctcagattaaatccatctcagaatc

cgctgatggaaacacagtgaatgctagattgtccagcgtgtccccaggacaaggtaaggactctcccgcga

APPENDIX D

***Streptomyces* strains* used in part II of this study for analyses of cellular fatty acids.**

Isolate #	SCPFRC strain	Isolated from:	Isolate #	SCPFRC strain	Isolated from:
1	M-2C-3	Soil	27	KV-D-6	Potato
2	M-7A-16	Soil	28	KV-Sh-1	Potato
3	M-7A-18	Soil	29	S-Sh-B1	Potato
4	M-2C-5	Soil	30	S-Sh-B6	Potato
5	B-B16	Soil	31	S-D-B2	Potato
6	B-B57	Soil	32	S-D-B7	Potato
7	B-B83	Soil	33	M-2A-3	Soil
8	B-B58	Soil	34	M-2C-4	Soil
9	M-D-2	Potato	35	M-7A-21	Soil
10	M-Sh-3	Potato	36	B-B14	Soil
11	M-Sh-10	Potato	37	B-B37	Soil
12	SP	Potato	38	B-B56	Soil
13	P-D-5	Potato	39	M-68	Soil
14	P-Sh-7	Potato	40	M-63	Soil
15	P-D-8	Potato	41	M-27	Soil
16	P-Sh-10	Potato	42	B-B82	Soil
17	B-Sh-1	Potato	43	B-B54	Soil
18	B-Sh-3	Potato	44	Co-4Sh-2	Potato
19	B -Sh-9	Potato	45	Co-4Sh-5	Potato
20	B-Sh-2	Potato	46	Co-1-1	Soil
21	RL 84-01-232	Potato	47	Co-1-2	Soil
22	DP	Potato	48	PR-3'-3	Potato
23	PonP	Potato	49	PR-3-2	Potato
24	Roy	Potato	50	LR90-3	Potato
25	KV-D-4	Potato	51	LM5MT-2	Potato
26	KV-Sh-3	Potato	52	LPit-2	Potato

*Source of *Streptomyces* strains is given in appendix B.

APPENDIX E

Streptomyces spp. entries in Actin 1 Database (ver. 3.7, January 1993):

S. albidoflavus
S. albus
S. antibioticus GC group A, B
S. californicus
S. cyaneus chartreusis
S. cyaneus coerulescens
S. cyaneus viridochromogenes A, B
S. exfoliatus
S. fradiae
S. glaucescens
S. griseoflavus
S. halstedii olivaceus
S. halstedii scabies
S. lavendulae
S. lydicus
S. rimosus
S. rochei rochei
S. rochei violaceoruber
S. violaceusniger hygrospicus hygrospicus
S. violaceusniger hygrospicus ossamyceticus A, B
S. violaceusniger violaceusniger

APPENDIX F
(from MIDI technical note #2, 1990)

The Euclidean distance (E-d) is the distance in two-dimensional space between two strains when their fatty acid compositions are compared. The unweighted pair-grouping method used in the MIS software seems to be very effective at comparisons at taxonomic levels of genus and lower, but should not be used for family comparisons.

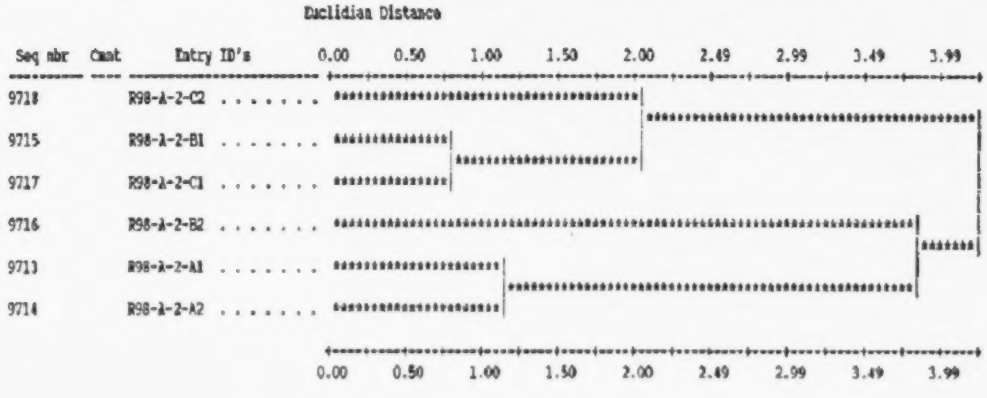
Dendrogram uses cluster analysis to produce unweighted pair matchings based on fatty acid compositions. The results are displayed graphically in a diagram that depicts the relatedness of pairs of entries. Multiple analyses of the same strain over a period of time results in linkages among samples of that strain at a level of 2 Euclidean distances or lower. Although the Euclidean distance scale permits quick determination of the relatedness of entries at the genus, species and subspecies (approximately 25, 10, and 6 Euclidean distances respectively), there is a strong dependence upon the current state of taxonomy in the taxa analysed.

APPENDIX G

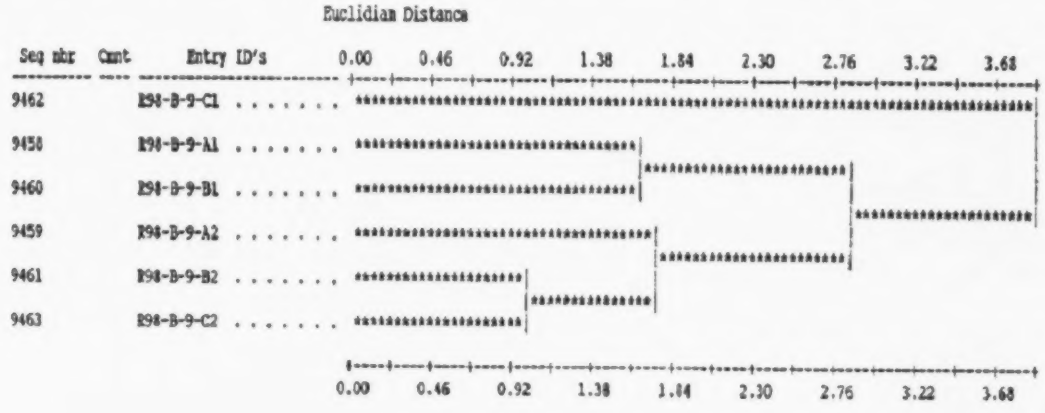
Comparisons of same-batch CFA profiles

These results indicated excellent repeatability of CFA profiles and confirmed they are of the same species and subspecies. Note: These were same-batch *Streptomyces* isolates grown under the new experimental conditions.

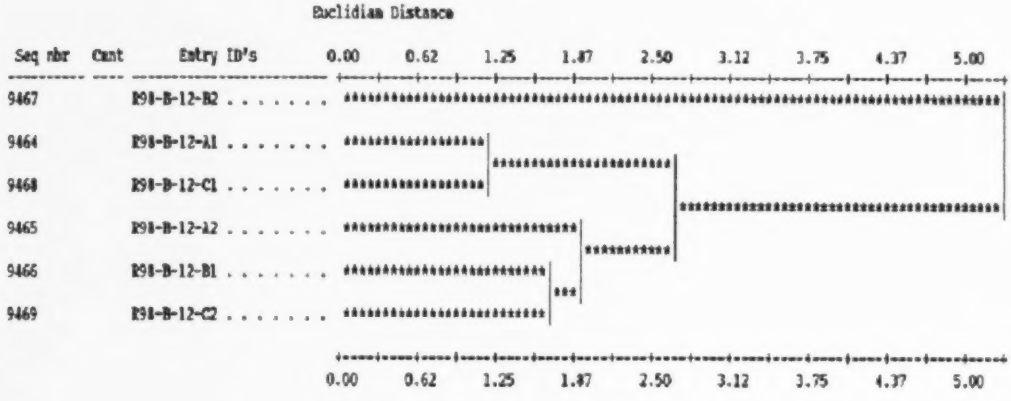
Dendogram (a): Shows approximately 4.24 E-d for six replicates of isolate R98-02.



Dendogram (b): Shows approximately 3.91 E-d for six replicates of isolate R98-09



Dendogram (c): Shows approximately 5.31 E-d for six replicates of isolate R98-12.

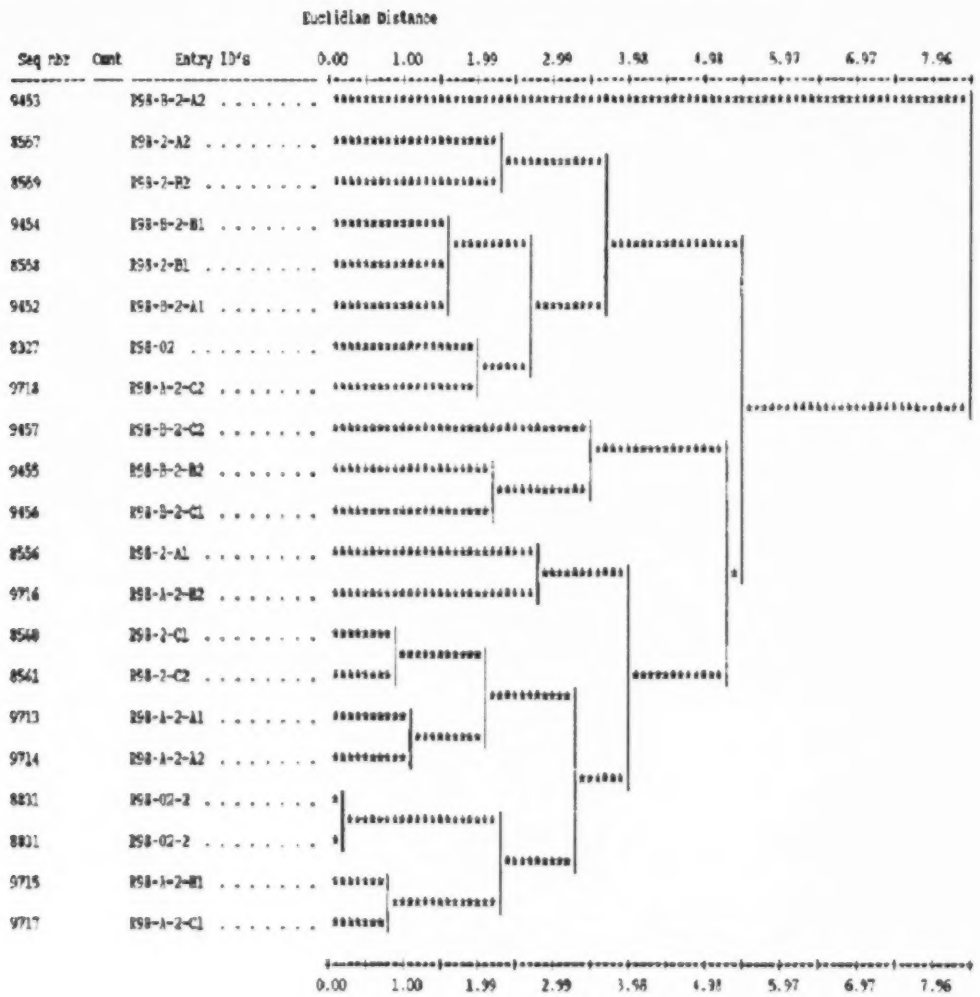


APPENDIX H

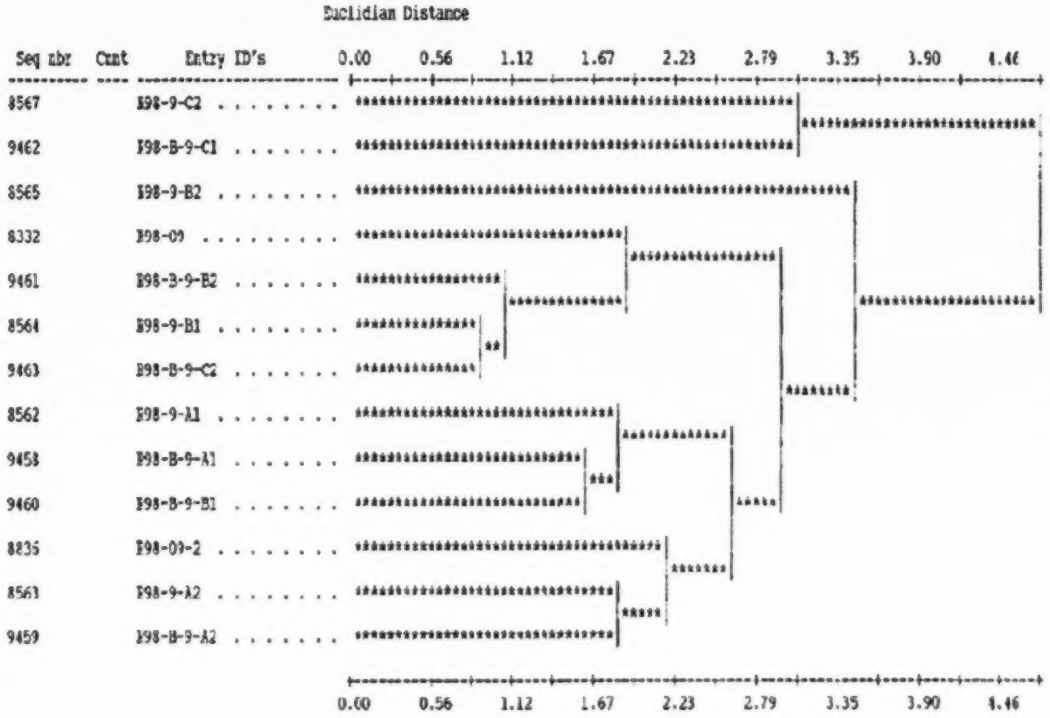
Comparisons of CFA profiles of *Streptomyces* grown over period of study

These results indicated excellent repeatability of CFA profiles and confirmed they are of the same species over the period of the study. Note: These *Streptomyces* isolates were grown under the new experimental conditions.

Dendogram (a): Shows approximately 8.46 E-d for replicates of isolate R98-02.



Dendogram (b): Shows approximately 4.74 E-d for replicates of isolate R98-09.



Dendogram (c): Shows approximately 8.73 E-d for replicates of isolate R98-12.